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Functional characterization of the *Escherichia coli* K-12 yiaMNO transport protein genes

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Summary

The yiaMNO genes of *Escherichia coli* K-12 encode a binding protein-dependent secondary, or tri-partite ATP-independent periplasmic (TRAP), transporter. Since only a few members of this family have been functionally characterized to date, we aimed to identify the substrate for this transporter. Cells that constitutively express the yiaK-S gene cluster metabolized the rare pentose L-xylulose, while deletion of the yiaMNO transporter genes reduced L-xylulose metabolism. The periplasmic substrate-binding protein YiaO was found to bind L-xylulose, and stimulated L-xylulose uptake by spheroplasts. These data indicate that the yiaMNO transporter mediates uptake of this rare pentose.

Keywords: *yiaMNO* genes, TRAP transporter, solute transport, functional characterization, L-xylulose.

Abbreviations: TRAP, tri-partite ATP-independent, ABC, ATP-binding cassette, TMD, transmembrane domain, pmf, proton-motive force, smf, sodium-motive force.

Introduction

Prokaryotes use a variety of transport proteins to take up solutes. Transporters are divided into distinct classes, based on the energy requirement of transport and the polypeptide composition (Driessen *et al.* 2000). Binding protein-dependent secondary transporters (Jacobs *et al.* 1996, Driessen *et al*. 1997, 2000), or tri-partite ATP-independent periplasmic (TRAP) transporters (Forward *et al.* 1997, Rabus *et al*. 1999, Kelly and Thomas 2001) form a new class of transporters that shares characteristics both with ATP-binding cassette (ABC) (Higgins *et al.* 1986, Ames and Joshi 1990, Higgins 1992) and secondary (Poolman and Konings 1993, Maloney and Wilson 1996) transporters. Transport involves an extracytoplasmic solute-binding protein, but the driving force is provided by the proton- (pmf) and/or sodium ion motive force (smf) rather than ATP hydrolysis. The membrane domain consists of a large sub-unit of 12 putative transmembrane domains (TMDs), resembling classical secondary transporters, and a small sub-unit of four putative TMDs (Driessen *et al.* 1997, Rabus *et al*. 1999, Wyborn *et al.* 2001). These transporters are found in all bacterial sub-divisions as well as in archaea (Driessen *et al*. 1997, Forward *et al.* 1997, Rabus *et al.* 1999, Kelly and Thomas 2001). Their architecture and biochemical characteristics pose intriguing mechanistic and evolutionary questions (Driessen *et al*. 2000).

Only a limited number of these systems has been described in molecular detail. Members are involved in transport of glutamate in *Rhodobacter sphaeroides* (Jacobs *et al.* 1996), C_4-dicarboxylate (malate, succinate, fumarate) in *R. capsulatus* (Forward *et al*. 1997) and *Wolinella succinogenes* (Ullmann *et al.* 2000), and ectoine and hydroxyectoine in *Halomonas elongata* (Grammann *et al.* 2002). The *Escherichia coli* K-12 *yiaMNO* genes encode one member of this transport protein family (Blattner *et al.* 1997; see also http://www-biology.ucsd.edu/ ~ msaier/transport/phylo/trap.html). The genes encoding the YiaMNO transporter are located within the *yiaK-S* gene cluster (GenBank accession nr. g1789999-08, Figure 1(*a*)) that has been implicated in carbohydrate utilization (Badia *et al*. 2000, Ibañez *et al*. 2000a). Strain JA134 constitutively expresses this cluster, which enables it to grow on L-lyxose (Sanchez *et al*. 1994, Badia *et al*. 2000). The gene located immediately downstream of *yiaMNO* encodes LyxK, a kinase that phosphorylates the pentose L-xylulose (L-*threo*-2-pentulose) (Badia *et al*. 1991, Sanchez *et al*. 1994). To provide more insight into the function of the YiaMNO transporter, we have carried out a deletion and biochemical analysis of the system in strain JA134. Our findings suggest that the YiaMNO transporter is involved in the uptake of L-xylulose.

Results

Deletion of the yiaMNO *genes*

An unmarked chromosomal deletion of the *yiaMNO* genes was constructed in strain JA134, yielding strain TP018. The deletion was confirmed by PCR (data not shown). The effect of the deletion was studied via RT-PCR, using primers directed against *yiaL*, *yiaM*, *yiaN*, *yiaO*, *lyxK* and *secY* (control). Only low-level expression of the *yiaL* and *yiaM* genes, but none of the other genes, was detected in the JA134 parental strain ECL1 (Figure 1(*b*)).

In strain JA134 all five genes were strongly expressed (Figure 1(*b*)) which is in agreement with previous observations (Ibañez *et al.* 2000a). Deletion of the *yiaMNO* genes did not affect transcription of the genes *yiaL* and *lyxK*, located immediately up- and downstream (Figure 1(*b*); TP018).

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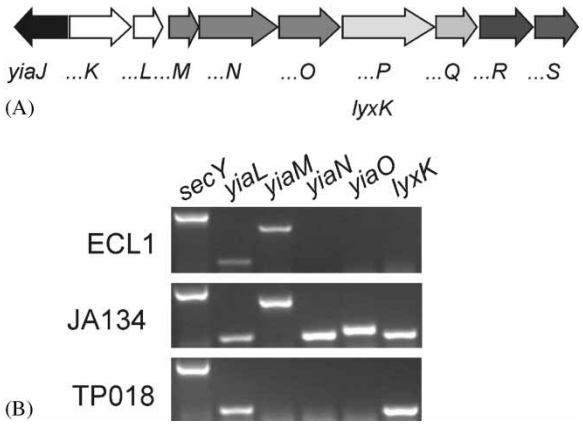

Figure 1. (*a*) Structural organization of the *yiaJ-S* gene cluster of *E. coli* K-12. *yiaJ*: putative regulator, *yiaK*: putative dehydrogenase, *yiaL*: unknown function, *yiaM*: small membrane domain of transporter (4 TMDs), *yiaN*: large membrane domain of transporter (12 TMDs), *yiaO*: periplasmic substrate-binding protein, *yiaP* (*lyxK*): kinase, phosphorylates both L-xylulose and 3-keto-L-gulonate, *yiaQ*: putative hexulose-6-phosphate synthase, *yiaR*: putative hexulose-6-phosphate isomerase, *yiaS*: ribulose-5-phosphate 4-epimerase (Blattner *et al.* 1997, Ibañez *et al.* 2000a,b, Yew and Gerlt 2002). (*b*) Deletion of the *yiaMNO* structural genes does not affect expression of up- (*yiaL*) and downstream (*lyxK*) genes. Expression of the *yiaL-lyxK* region in the three different strains was detected via RT-PCR. Primers were designed to detect mRNA fragments of *secY* (control for constitutive expression, lane 1), *yiaL* (lane 2), *yiaMNO* (the putative transporter, lanes 3–5), and *lyxK* (lane 6).

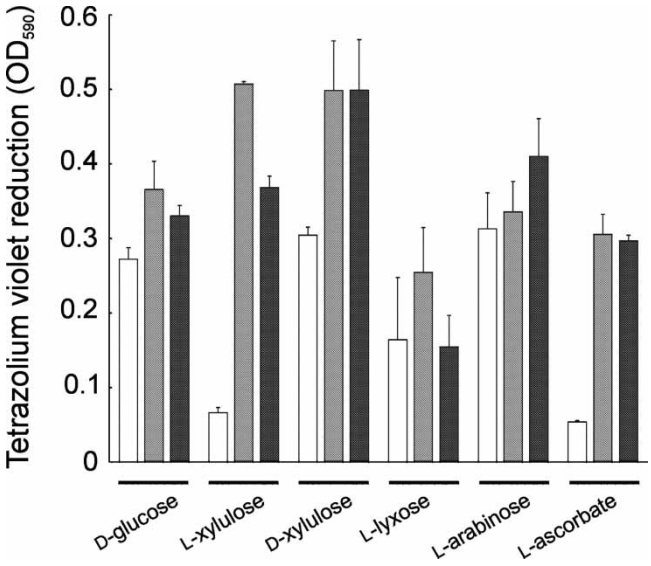

Figure 2. Only cells expressing the *yiaK-S* gene cluster metabolize L-xylulose, and deletion of the YiaMNO transporter negatively affects this property. Metabolic activity of the strains on various putative carbon sources was measured via tetrazolium violet reduction, which is expressed in OD_{590} values. ECL1: white bars, JA134: grey bars, and TP018: black bars. In the absence of carbon source no tetrazolium violet was reduced (not shown).

Carbon source metabolism by E. coli *K-12 strains ECL1, JA134 and TP018*

Strains ECL1, JA134 and TP018 were analysed for their ability to metabolize potential substrates of the YiaMNO transporter. For this purpose, the irreversible reduction of tetrazolium violet to its purple formazan was used as an indicator for carbon source catabolism (Bochner and Savageau 1977), using various test substrates.

L-lyxose was metabolized by strains expressing the cluster, but deletion of the *yiaMNO* genes had no significant effect (Figure 2, error bars). L-lyxose is a substrate for the L-rhamnose-H^+ symporter RhaT (Badia *et al*. 1991, Muiry *et al*. 1993), and *rhaT* deletion mutants of JA134 do not grow on L-lyxose (Badia and Aguilar, unpublished data). Thus, L-lyxose enters the cell via RhaT, and not via the YiaK-S system. A recent report showed that the kinase LyxK phosphorylates 3-keto-L-gulonate, a breakdown product of L-ascorbate metabolism or the result of the reduction of 2,3-diketo-L-gulonate (Yew and Gerlt 2002). L-ascorbate is a substrate for cells that constitutively express the cluster, but metabolism of this compound is not affected in strain TP018 (Figure 2). 2,3-diketo-L-gulonate, which is not commercially available, was not tested in these experiments, but its presence in L-ascorbate solutions at pH 7.0 has been reported (Simpson and Ortwerth 2000). However, no induction of the *yiaK-S* cluster by L-ascorbate-derived 2,3-diketo-L-gulonate, making use of the ϕ(*yiaK-lacZ*) in the genetic background of strain ECL1, was detected (data not shown). Therefore, 2,3-diketo-L-gulonate is probably not a substrate of the *yiaK-S* operon.

Expression of the *yiaK-S* cluster allowed JA134 to utilize L-xylulose, whereas ECL1 did not. Effects of the *yiaMNO* deletion were only observed on this pentose, as TP018 metabolized L-xylulose less efficiently than JA134 (Figure 2). However, deletion of the *yiaMNO* genes did not abolish L-xylulose metabolism (Figure 2); therefore, an additional L-xylulose-transporting system exists. Further work focused on L-xylulose as a potential substrate for the YiaMNO transporter.

L-xylulose is taken up by strains JA134 and TP018

Solute transport studies are most conveniently performed with radioactively labelled substrates. However, as L-xylulose is not available in radio-labelled form, we have made use of assays that allow measurement of L-xylulose consumption by whole cells in a non-radioactive manner. NADP-xylitol dehydrogenase can be used to monitor indirectly the L-xylulose concentration (see Experimental procedures, Ashwell 1984). Strains ECL1, JA134 and TP018 were incubated in the presence of L-xylulose as the sole carbon and energy source. In contrast to wild-type strain ECL1, strain JA134 rapidly metabolized L-xylulose (Figure 3).

The *yiaMNO* deletion strain TP018 was capable of utilizing L-xylulose, but reproducibly at a lower initial rate than strain JA134 (Figure 3). Thus, L-xylulose is taken up and metabolized by strains that constitutively express *yiaK-S*. The YiaMNO transporter is involved in, but not essential for L-xylulose utilization in this genetic background.

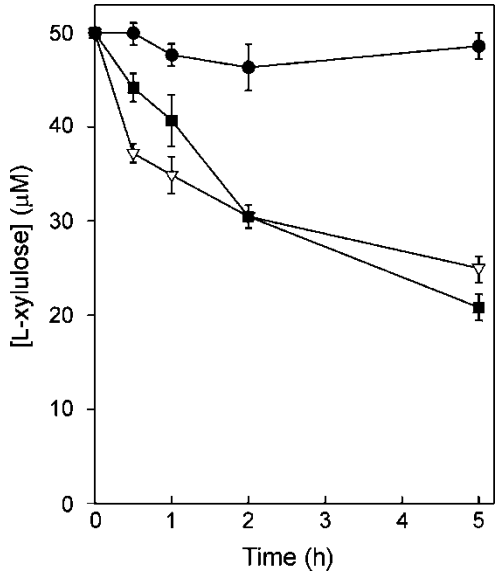

Figure 3. Uptake of L-xylulose by whole cells. The extra-cellular L-xylulose concentration was monitored over time for strains ECL1 (black circles), JA134 (white triangles), and TP018 (black squares). ECL1 is unable to metabolize L-xylulose, while strain JA134 rapidly consumes the pentose. The initial uptake ($t = 0-1$ h) of TP018 lags behind that of JA134, but eventually both activities are nearly indistinguishable.

YiaO binds L-xylulose and stimulates metabolism by spheroplasts expressing yiaMN

The enzyme-based reaction was also used to measure L-xylulose transport and binding. The binding protein YiaO was over-expressed with a C-terminal 6xHis-tag and purified to homogeneity by Ni^{2+}-NTA-affinity chromatography (data not shown). Purified YiaO was incubated with substrate and then filtered from the solution. The L-xylulose concentration of the flow through was determined. Approximately 10% of the L-xylulose had been removed through binding by YiaO (Figure 4).

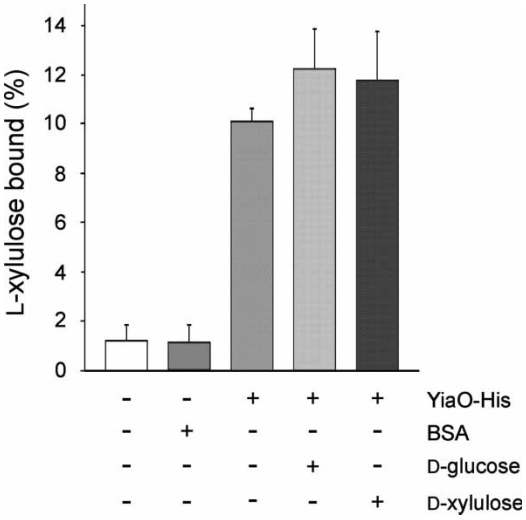

Figure 4. Purified YiaO-His specifically binds L-xylulose. Percentage of L-xylulose bound by YiaO-His in the retention assay, in the presence (+) or absence (−) of the potential competitors D-glucose and D-xylulose. BSA: control for non-specific binding to protein.

No non-specific binding of L-xylulose to either the filter or bovine serum albumin (BSA, Figure 4) was observed. Binding of L-xylulose was not competed by a 20-fold excess of either D-glucose or D-xylulose (Figure 4). Although this method is unsuitable for estimating the dissociation constant for binding, the data indicate that YiaO is able to bind L-xylulose.

Spheroplasts prepared from strains ECL1, JA134 and TP018 were incubated with L-xylulose in the absence and presence of purified YiaO-His. Following incubation, the spheroplasts were removed by centrifugation, and the concentration of the L-xylulose remaining in the supernatant was determined. ECL1 spheroplasts did not metabolize L-xylulose (Figure 5).

Spheroplasts of strains JA134 and TP018 both consumed L-xylulose in the absence of YiaO-His. However, stimulation of L-xylulose utilization was observed only upon addition of purified YiaO-His to JA134 spheroplasts, but not TP018 which lacks the transporter genes (Figure 5). Taken together, these experiments show that YiaO-His is able to bind L-xylulose, and together with the YiaMN proteins can function as a binding protein-dependent uptake system for L-xylulose.

Discussion

The *yiaMNO* genes of *E. coli* K-12 encode a putative binding protein-dependent secondary, or TRAP, transporter (Driessen *et al*. 1997, Forward *et al.* 1997, Rabus *et al.* 1999, Kelly and Thomas 2001). In order to investigate the role of the YiaMNO transporter, *E. coli* K-12 strain MC4100 and the

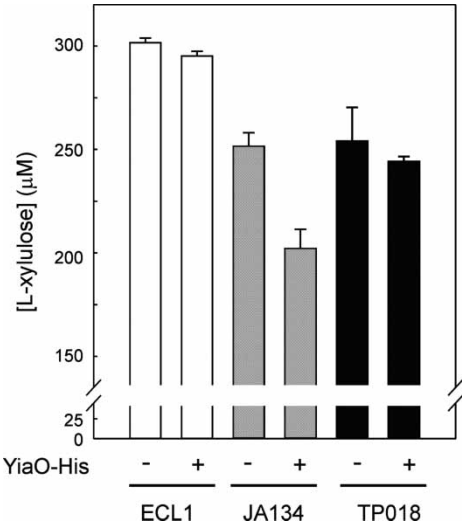

Figure 5. YiaO-His stimulates L-xylulose uptake by spheroplasts expressing *yiaMN*. L-xylulose uptake, in the absence (−) and presence (+) of YiaO-His, by spheroplasts prepared from the three strains was determined by measuring the concentration of the pentose in the supernatant after incubation. ECL1 (white bars) does not take up the pentose. Both JA134 (grey bars) and TP018 (black bars) spheroplasts take up L-xylulose, but the addition of YiaO-His increases this activity only in the case of JA134, which expresses *yiaM* and *yiaN*.

Δ*yiaMNO* derivative TP001 have been used in an extensive screen, but were found indistinguishable with over 100 substrates tested (see Experimental procedures). However, the use of mutant strain JA134 resulted in identification of one substrate for the transporter. Whole cells utilized L-xylulose only when the *yiaK-S* cluster was constitutively expressed (Figure 2), and the initial L-xylulose metabolic activity was lowered when the YiaMNO transporter was deleted (Figure 3). Interestingly, there must be a second L-xylulose transporting system present in these strains, but its activity is detected only in cells expressing the metabolic enzymes (see below).

L-xylulose transport has not been studied before, mainly because this compound is not available in radio-labelled form. Evidently, this also hampered the analysis presented in this report. Therefore, indirect binding and transport assays were used. YiaO, the periplasmic substrate-binding protein of the transporter, specifically binds L-xylulose (Figure 4), and stimulated metabolism of the pentose by spheroplasts only when the membrane domains YiaMN were expressed (Figure 5). Taken together, these findings demonstrate that the YiaMNO transporter is capable of mediating L-xylulose uptake. However, we cannot exclude that the major role of this transporter concerns the uptake of other pentoses as the YiaMNO system is neither induced by L-xylulose (Badia and Aguilar, unpublished data), nor do wild-type cells grow on L-xylulose.

The second L-xylulose transporting system is most likely also present in the wild-type strain ECL1, but its activity escapes detection owing to the absence of L-xylulose-metabolizing enzymes. Possible L-sugar uptake systems of *E. coli* K-12 that may be involved in this activity are RhaT (Tate *et al*. 1992) that recognizes the structurally related sugar L-lyxose (Muiry *et al*. 1993), or FucP (Gunn *et al*. 1994) that transports L-fucose, L-galactose and D-arabinose (Muiry *et al*. 1993). The specificity of these transporters, however, is determined by the nature of the side-chains at both the C-2 and the C-4 positions of the pyranose rings (Muiry *et al*. 1993) while L-xylulose forms a furanose ring. Moreover, L-xylulose does not compete with L-rhamnose for transport (Badia and Aguilar, unpublished data). Therefore, the identity of the second systems remains to be determined.

The mechanistic properties of the YiaMNO transporter need further investigation, but, since there is no radio-labelled L-xylulose available, these studies are extremely difficult to perform at this time. In addition, the physiological function of L-xylulose uptake and metabolism by *E. coli* K-12 is unclear. Binding protein-dependent secondary transporters have previously been implicated in the uptake of organic anions and compatible solutes. Our report extends the substrate range to a pentose sugar.

Experimental procedures

Bacterial strains, plasmids, primers and growth conditions

Escherichia coli K-12 strains, vectors and recombinant plasmids used in this study are listed in Tables 1 and 2, respectively. PCR and RT-PCR primers are listed in Table 3.

EC1000 was used for handling pORI240 and its derivatives, and SF100 was used for over-expression of His-tagged YiaO. Cells were grown aerobically at 37°C in Luria Broth (LB) or in M63 minimal medium (Atlas 1993). Antibiotics were added to final concentrations: ampicillin (Am), 50 μg/ml; tetracyclin (Tc), 12 μg/ml; kanamycin (Km), 50 μg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was used at 30 μg/ml.

Table 1. *E. coli* K-12 strains used in this study.

Strain	Characteristics	Source or reference
MC4100	*araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Laboratory collection
TP001	MC4100 Δ*yiaMNO*	This work
ECL1	HfrC *phoA8 relA1 tonA22* $T2^r$ (λ)	Lin (1976)
$JA134^+$	ECL1 *lyx*	Sanchez *et al.* (1994)
TP018	JA134 Δ*yiaMNO*	This work
EC1000	MC1000 $repA^+$	Leenhouts *et al.* (1996)
SF100	KS272 Δ*ompT*	Baneyx and Giorgiou (1990)
XL1-Blue	*recA*1 *lac hsdR17 supE44 relA1* (F' *proAB* $lacI^q$ *lacZ* dM15 Tn*10)*	Strategene
TE2680	F^- λ^- IN (*rrnD-rrnE*) Δ(*lac*)*X74 rplS galK2* *recD::*Tn*10d-tet trpDC700::put-PA1303*: [Km^s Cm^r *lac*]	Elliott (1992)

Table 2. Plasmids used in this study.

Plasmid	Characteristics	Source or reference
pET401	Cloning vector, Am^r, *trc*-promoter	Van der Does *et al.* (1998)
pET908	pET401 carrying fused F1-F2 flanking regions	This work
pORI240	Tc^r, $LacZ^+$, ori^+ of pWV01, requires *repA in trans* for replication	Leenhouts *et al.* (1996)
pORIF1F2	pORI240 carrying fused *yiaMNO*-flanking regions	This work
pRS550	Promoter-less *lac*, Km^r, Am^r	Simons *et al.* (1987)
pSA5	Expression vector, Am^r, C-terminal 6xHis-tag	S.V. Albers
pET917	pSA5 carrying *yiaO*	This work

Search for the substrate of YiaMNO

The following compounds were tested as substrates of the transporter in growth experiments, metabolic assays (tetrazolium violet) and, when available in radioactive form, in transport and binding experiments: (miscellaneous) Tween-20, Tween-40, Tween-80; (C_{18}) maltotriose; (C_{12}) cellobiose, α-D-lactose, lactulose, maltose, D-melibiose, sucrose, D-trehalose; (C_{10}) adenosine, 2'-deoxy adenosine, inosine, thymidine; (C_9) uridine; (C_8) N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, *m*-hydroxy phenylacetic acid, *p*-hydroxy phenylacetic acid, phenylethylamine, tyramine; (C_7) glycyl-L-glutamic acid, glycyl-L-proline, α-methyl galactoside, β-methyl glucoside; (C_6) L-ascorbate, Fe_3-citrate, iso-citrate, 2,3-diketo-L-gulonate, dulcitol, ectoine, D-fructose, fructose-6-phosphate, L-fucose, D-galactonic acid γ-lactone, L-galactonic acid γ-lactone, D-galactose, D-galacturonic acid, D-gluconate, D-glucosaminic acid, D-glucose, glucose-1-phosphate, glucose-6-phosphate, glucuronamide, D-glucuronic acid, glycyl-L-aspartic acid, α-hydroxy glutaric acid γ-lactone, *m*-inositol, D-mannitol, D-mannose, mucic acid, D-psicose, L-rhamnose, D-saccharic acid, D-sorbitol, tricarbalyllic acid; (C_5) adonitol, L-alanyl-glycine, L-arabinose, L-glutamate, L-gluta-

Table 3. Primers used for PCR and RT-PCR.

Gene/fragment	Sequence	Forward/Reverse	Site[a]
Flanking region [b] 1	CCCTGAATTCATTCAACAACTGGA	F	*Eco*RI
Flanking region [b] 1	AAGTCGGATCCGCAGCAACCTG	R	*Bam*HI
Flanking region[b] 2	AGGGATCCGATCTGCTGAAAGCC	F	*Bam*HI
Flanking region[b] 2	CATATCTAGAAGCAGGGTGCGCA	R	*Xba*I
Deletion check	GATACCGGGCAGCTACGC	F	
Deletion check	ATACCGACAATTTGTTCCC	R	
secY, fragment[c]	GGCCTGGTGATTAACCCG	F	
secY, fragment[c]	CCGAATTCCTGGTACAAATGC TCCGGACTTCTT	R	
yiaL, fragment[d]	CTACCGAATTCAACGCCCTGGAGC	F	
yiaL, fragment[d]	TTGCGCGGATCCAATAGTGAT	R	
yiaM	TAGCCATGGAAAAAATACTCGAAGC	F	*Nco*I
yiaM	GGGGAATTCTTAAGCTCCTTGCGG	R	*Eco*RI
yiaN, fragment[d]	CCGATTGAATTCGGTGTCATG	F	
yiaN, fragment[d]	GCGGGATCCTTAATCCATTTCAAAGGG	R	
yiaO	GGGCCATGGAATTACGCTCTGTAACC	F	*Nco*I
yiaO	CCCGGATCCACCTTGCACCTCATCCAC	R	*Bam*HI
yiaO, fragment[d]	AAGTCCGGAATTCCAGCAGGC	F	
yiaO, fragment[d]	CCCTCTAGATTATTGCACCTCATCCAC	R	
lyxK, fragment[d]	AATACTGGATCCGGTTAGATTGTGG	F	
lyxK, fragment[d]	CCGAGAATTCGTTCCCCGCTAACA	R	
yiaK, promoter	TCCCCATTTTGTCGCGTCCTG	F	
yiaK, promoter	ACGCCGCGTGAAATTAAGAC	R	

[a]restriction endonuclease site introduced by primer
[b]1,620 bp up- (1) and downstream (2) regions flanking *yiaMNO*
[c]632-bp internal fragment
[d]200-bp internal fragment

mine, glycine betaine, α-keto butyric acid, α-keto glutarate, L-lyxose, mono-methyl succinic acid, L-proline, D-ribose, D-xylose, D-xylulose, L-xylulose; (C_4) acetoacetic acid, L-asparagine, D-aspartate, L-aspartate, bromo succinic acid, fumarate, α-hydroxy butyric acid, D-malate, L-malate, methyl pyruvate, oxaloacetic acid, succinate, *m*-tartaric acid, D-threonine, L-threonine; (C_3) D-alanine, L-alanine, glycerol, D,L-α-glycerol phosphate, L-lactic acid, malonate, propionic acid, pyruvic acid, 1,2-propanediol, D-serine, L-serine; (C_2) acetic acid, 2-amino ethanol, glycolate, glyoxylate; (C_1) formic acid. Transport experiments using radio-labelled compounds were performed according to the rapid filtration method (Lolkema *et al.* 1994).

Chromosomal deletion of the yiaMNO *operon*

Upstream (F1) and downstream (F2) flanking regions were cloned as 1620 bp PCR-fragments introducing *Bam*HI-sites for fusion in pET908. The F1/F2-fragment was cloned into pORI240, creating pORIF1F2. Using this vector, an unmarked chromosomal deletion of the *yiaMNO* genes in *E. coli* strains MC4100 and JA134 was created according to Leenhouts *et al.* (1996). The deletion was checked via PCR, and mutants were labelled TP001 and TP018, respectively.

Isolation of total RNA and RT-PCR

Cells were grown in LB medium to an OD_{660} of 1.0, harvested and frozen in liquid nitrogen. Lysozyme (20 μg/ml) was added at room temperature (RT). Pellets were dissolved in 1 ml Trizol reagent (GIBCO BRL) and incubated at RT for 5 min. Chloroform (200 μl) was added, the suspension was mixed ($>$ 15 sec), allowed to settle at RT for 3 min, and centrifuged (12 000 × g, 15 min, 4°C). The aqueous phase was transferred to a new tube, isopropanol was added, followed by mixing. Samples were incubated at RT (10 min) and centrifuged as above. Pellets were washed twice with 70% (v/v) ice-cold ethanol (7500 × g, 5 min, 4°C), air-dried and re-suspended in a small volume of RNase-free demineralized water. Concentrations were determined using the Gene Quant system (Amersham Pharmacia Biotech). RT-PCR was performed with RT-PCR-beads (Amersham Pharmacia Biotech) following manufacturer's instructions, using 1 μg total RNA per reaction. Primers detected 200-bp internal fragments, except *yiaM* (474-bp complete fragment) and *secY* (632-bp internal fragment). Products were analysed on ethidium bromide-stained 2% agarose gels.

Construction of the yiaK-lacZ *transcriptional fusion*

The 268-bp 5'-upstream region of the *yiaK* gene was amplified by PCR and transcriptionally fused to *lacZ* by insertion into plasmid pRS550 (Simons *et al*. 1987). Recombinant plasmids were selected after transformation of strain XL1-Blue, and the correct orientation was confirmed by sequencing using an M13 primer. Merodiploids were obtained by transferring the fusions as single copies into the *trp* operon of *E. coli* strain TE2680 as described (Elliott 1992). Transformants were selected for Km resistance and sensitivity to Am and Chloroamphenicol. P1 *vir* lysates were made to transduce the fusions into strain ECL1.

Tetrazolium violet metabolic assays

Prefabricated ES Microplates (Biolog) were used following the manufacturer's instructions to test carbon source usage by strain MC4100 and its Δ*yiaMNO* derivative TP001. Metabolic properties of strains ECL1, JA134 and TP018 were investigated using the redox indicator tetrazolium violet (Sigma) (Bochner and Savageau 1977). Assays were performed in sterile 96 wells microplates (Greiner) with 150 μl per well. Tetrazolium violet and carbon sources were added to final concentrations of 0.0025% and 0.2% (w/v), respectively. Cells were scraped from a LB-agar plate, re-suspended in M63 minimal medium and added to the wells. Plates were incubated overnight at 37°C. Absorbance at 590 nm was measured using a Spectramax 340 titertek-reader (Molecular Devices).

Enzymatic detection of L-xylulose

NADP-xylitol dehydrogenase catalyses the stereo-specific reaction (Hickman and Ashwell 1959):

$$\text{L-xylulose} + \text{NADPH} + \text{H}^+ \rightarrow \text{xylitol} + \text{NADP}^+$$

The decrease in absorbance at 340 nm due to consumption of NADPH is proportional to the L-xylulose concentration (Ashwell

1984). NADP-xylitol dehydrogenase was isolated from 5 g of acetone-dried guinea-pig liver (Sigma) by a modification of the method of Ashwell (1984), and finally dissolved in 5 ml demineralized water. Aliquots were frozen in liquid nitrogen and stored at −80°C.

In the assay, to a final volume of 1800 μl were successively added: 75 mM Tris-HCl, pH 7.0; 5 mM $MgCl_2$; 1 mM cysteine-HCl; 100 μM β-NADPH; 50–100 μl enzyme suspension. After stabilization of the signal, 200 μl sample with unknown L-xylulose content was added. Measurements were performed at 37°C under continuous stirring, using a spectrophotometer (Cary). A calibration curve was used, which was linear in the range of 10–60 μM (Ashwell 1984).

Over-expression and purification of YiaO

The *yiaO* gene was cloned via PCR and ligated into pSA5, yielding vector pET917, encoding YiaO with a carboxyl-terminal 6xhistidine tag. *Escherichia coli* SF100 was transformed with pET917, grown to an OD_{660} of 0.6, and over-production was induced by addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). Cells were harvested (4000 × g, 10 min, 4°C). The periplasmic fraction was isolated using the cold osmotic shock procedure (Neu and Heppel 1965), supplemented with 50 mM potassium phosphate, pH 7.4; 100 mM NaCl; 15 mM imidazole, and incubated overnight with Ni^{2+}-NTA agarose (Qiagen) at 4°C. Unbound material was eluted, the column was washed twice with buffer (50 mM potassium phosphate, pH 7.4; 100 mM NaCl; 15 mM imidazole), and YiaO-His was eluted in the same buffer containing 200 mM imidazole. Fractions were analysed on 15% SDS-PAGE, by Coomassie Brilliant Blue (CBB) and silver staining.

Substrate binding assay

L-xylulose binding by YiaO-His was studied by modifying a substrate retention assay (Tetsch and Kunte 2002). One hundred μM purified YiaO-His or BSA, was incubated with 50 μM L-xylulose for 10 min at 37°C. D-glucose and D-xylulose were used at 1 mM final concentration. Protein was removed by centrifugation (10000 × g, 45 min, 4°C) using Microcon YM-10 (10 kDa cut-off) filters. The flow-through was analysed using the L-xylulose detection assay.

Utilization of L-xylulose

For whole-cell and spheroplast uptake experiments, strains ECL1, JA134 and TP018 were grown in LB to an OD_{660} of 1.0, and harvested as above. Cells were re-suspended to an OD_{660} of 5 in M63 containing L-xylulose (0.5 mM final concentration), and incubated at 37°C under continuous shaking. Samples were taken at various intervals. Cells were removed by centrifugation (13000 × g, 5 min, RT) and the supernatant (200 μl) was analysed for L-xylulose content. Spheroplasts were prepared as described by Cao *et al.* (1994). Three hundred-microlitre spheroplasts (10 mg/ml final concentration) in 50 mM potassium phosphate, pH 7.4; 5 mM $MgSO_4$, were mixed with 100 μl YiaO-His (1 mg/ml), pre-incubated with 1.2 mM L-xylulose for 5 min at RT, or buffer. The suspension was shaken at 37°C for 30 min, followed by centrifugation (13000 × g, 5 min, RT), and supernatants was analysed for the L-xylulose content.

Other methods

β-galactosidase activity was assayed by hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and expressed as Miller Units (Miller 1992). Protein content was determined using the DC protein assay (Biorad).

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